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IN VITRO HEPATOTOXICITY OF SINTHETIC CATHINONES AND BENZOFURANS USED AS 'LEGAL HIGHS'

Dissertação de 2º ciclo de estudos conducente ao grau de Mestre em Toxicologia Analítica Clínica e Forense

Trabalho realizado sob a orientação da Doutora Diana Cristina Dias da Silva e da Professora Doutora Helena Maria Ferreira da Costa Ferreira Carmo

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The following comunications were presented at scientific meetings:

1. Rita Roque Bravo, Maria João Martins, Ana Pinto, Maria João Valente, Helena Carmo, Diana Dias da Silva (2016) In vitro hepatotoxicity of six synthetic cathinones and benzofurans that have been misused an ‘legal highs’ or ‘smart drugs’.

2. Rita Roque Bravo, Maria João Martins, Ana Pinto, Maria João Valente, Paula Guedes, Félix Carvalho, Maria de Lourdes Bastos, Helena Carmo, Diana Dias da Silva (2016) ‘Legal highs’ and ‘Smart drugs’: hepatotoxicity of six emerging club drugs

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Abstract

In the past few years, the world has witnessed the surge of new psychoactive substances (NPS) and the rise in their popularity within the club scene. The interest in these substances derived from their capacity to elicit similar effects to those of classic stimulants such as cocaine and amphetamines, with the advantages of the relative ease to obtain and the legal status. This fostered the unregulated rise of substances like mephedrone and 3,4-methylenedioxypyrovalerone (MDPV), but recent legislation has also banned these drugs in several countries. Consequently, new synthetic phenethylamines appeared on the drug market, untested for human safety.

This works aimed to investigate the cytotoxic potential of four synthetic cathinones (butylone, buphedrone, 3,4-dimethylmethcathinone [3,4-DMMC] and 4-fluoromethcathinone [4-FMC]) and two benzofurans (6-(2-aminopropyl)benzofuran [6-APB] and 5-(2-aminopropyl)benzofuran) [5-APB] that have recently emerged in the described context.

When considering the toxicological assessment of new drugs, the liver is an organ that stands out for its pivotal role in the metabolism of these toxicants; therefore, we chose three different *in vitro* models for the hepatocyte to accomplish our aim: primary rat hepatocytes and the immortalized cell lines HepaRG and HepG2. Concentration-response curves for all drugs were established in the three cell models, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; cells models were exposed for 24h to a wide range of concentrations. Then, a set of oxidative stress endpoints and energetic status were evaluated in primary rat hepatocytes, by testing each drug at the respective EC_{20}, EC_{40}, EC_{50} and EC_{70}.

For all *in vitro* models, 3,4-DMMC was the most toxic substance (EC_{50} 0.16 mM in primary rat hepatocytes; 0.7 mM in HepaRG cells; and 0.62 mM in HepG2 cells). 4-FMC was the least toxic drug for primary rat hepatocytes (EC_{50} 2.21 mM) and buphedrone was the least toxic compound for the cell lines (EC_{50} 9.62 mM and 13.67 mM for HepaRG and HepG2 cells, respectively). Additionally, 3,4-DMMC and 4-FMC elicited a notorious increase in the production of reactive oxygen and nitrogen species; all substances were capable of inducing a concentration-dependent shortage in antioxidant defences (glutathione) and of interfering with the normal energetic status.

Our work provides the first evidence of the hepatotoxic potential of these new psychoactive substances and sheds some light on the molecular mechanisms underlying the observed effects.

Keywords: hepatocytes, cytotoxicity, oxidative stress, synthetic cathinones, 'benzofury'

Resumo

Nos últimos anos, o mundo testemunhou o surgimento de novas substâncias psicoativas (NPS) e o aumento da sua popularidade dentro do ambiente de discoteca. O interesse nestas substâncias prende-se com a sua capacidade de provocar efeitos semelhantes aos de estimulantes clássicos, como a cocaína e as anfetaminas, com as vantagens da relativa facilidade de obtenção e o estatuto legal. Isto fomentou o aumento descontrolado de substâncias como a mefedrona e 3,4-metilenodioxipirovalerona (MDPV), mas a legislação recente proibiu também estas drogas em vários países. Como consequência, novas feniletilaminas sintéticas apareceram no mercado de drogas, sem que tenham sido testadas para a segurança humana.

O objetivo deste trabalho foi investigar o potencial citotóxico de quatro catinonas sintéticas (butilona, bufedrona, 3,4-dimetilmetcatinona [3,4-DMMC] e 4-fluorometcathinona [4-FMC]) e dois benzofuranos (6-(2-aminopropil)benzofurano [6-APB] e 5-(2-aminopropil)benzofurano) [5-APB] que surgiram recentemente no contexto descrito.

Quando se considera a avaliação toxicológica de novas drogas, o fígado é um órgão que se destaca pelo seu papel central no metabolismo destas substâncias tóxicas; portanto, nós selecionámos três modelos *in vitro* diferentes de hepatócito para alcançar o nosso objetivo: hepatócitos primários de rato e as linhas celulares imortalizadas HepaRG e HepG2. As curvas de concentração-resposta para todas as drogas foram determinadas nos três modelos de células, utilizando o ensaio de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio (MTT); os modelos celulares foram expostos durante 24 horas para uma vasta gama de concentrações. De seguida, um conjunto de marcadores de stress oxidativo e do estado energético foram avaliados nos hepatócitos primários de rato, pelo ensaio de cada fármaco nas respetivas EC_{20}, EC_{40}, EC_{50} e EC_{70}.

Para todos os modelos *in vitro*, a 3,4-DMMC foi a substância mais tóxica (EC_{50} 0,16 mM em hepatócitos primários de rato; 0,7 mM na linha celular HepaRG; e 0,62 mM na linha celular HepG2). A 4-FMC foi a droga menos tóxica para os hepatócitos primários de rato (EC_{50} 2,21 mM) e a bufedrona o composto menos tóxico para as linhas celulares (EC_{50} 9,62 mM e 13,67 mM nas linhas celulares HepG2 e HepaRG, respetivamente). Para além disso, 3,4-DMMC e 4-FMC despoletaram um aumento notório na produção de espécies reativas de oxigénio e de azoto; todas as substâncias foram capazes de induzir uma escassez dependente da concentração das defesas antioxidantes (glutationa) e de interferir com o estado energético normal.

O nosso trabalho fornece a primeira evidência do potencial hepatotóxico destas novas substâncias psicoativas e elucida os mecanismos moleculares subjacentes aos efeitos observados.

Palavras-chave: hepatócitos, citotoxicidade, stress oxidativo, catinonas sintéticas, “benzofury”

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Abbreviations

3,4-DMMC – 3,4-dimethylmethcathinone

3-FMC – 3-fluoromethcathinone

4-FMC – 4-fluoromethcathinone

5-APB – 5-(2-aminopropyl)benzofuran

5-HT - Serotonin

6-APB – 6-(2-aminopropyl)benzofuran

ATP – Adenosine Triphosphate

BBB – Blood-Brain Barrier

CAR – Constitutive Androstane Receptor

COMT – Catechol O-methyl Transferase

CYP – Cytochrome P450

DA – Dopamine

DAT – Dopamine Transporter

DCFH-DA – 2',7'-dichlorodihydrofluorescein diacetate

DTNB - 5,5-dithio-bis(2-nitrobenzoic acid)

EC – Effect Concentration

EGTA – Ethylene Glycol Tetraacetic Acid

EMCDDA – European Monitoring Centre for Drug and Drug Addiction

GSH - Glutathione

GSSH – Oxidized Glutathione

MAO – Monoamine Oxidase

MDMA – 3,4-methylenedioxymethamphetamine

MDPV – 3,4-methylenedioxypyrovalerone

MDR1 – Multudrug Resistance Protein 1

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NA – Noradrenaline

NAT – Noradrenaline Transporter

OCT – Organic Cation Transporter

NPS – New Psychoactive Substances

PXR – Pregnane X Receptor

RNS – Reactive Nitrogen Species

ROS – Reactive Oxygen Species

SERT – Serotonin Transporter

TAAR1 – Trace Amine-Associated Receptor 1

Introduction

New psychoactive substances (NPS) have been on the rise since their introduction in the drug markets, with the number of new substances and seizures increasing at alarming rates. The 2016 European Drug Report points that, in 2015, 98 new drugs were detected, which accounts for a total of 560 NPS monitored by de European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 70% of which have appeared in the last 5 years (EMCDDA, 2016). Two of the most prominent groups of NPS are synthetic cathinones and benzofurans. As popularity of novel derivatives increased, the number of intoxications related to the substances of these groups, including some deaths, has risen (Adamowicz et al., 2014; Batisse et al., 2014; Borek and Holstege, 2012; Carbone et al., 2013; Chan et al., 2013; Kramer et al., 2016; Pearson et al., 2012; Thornton et al., 2012; Wood et al., 2010; Wright et al., 2013) but their toxicological spectrum are far from being fully elucidated and information on the molecular mechanisms responsible by the detrimental effects of NPS is yet to be disclosed.

Therefore, to provide some potential insight into the reasons behind random occurrence of extreme toxicity after consumption of these NPS and to increase the knowledge necessary to aid diagnostics and treatment of intoxications, our group sought to investigate the hepatotoxic effects of a few synthetic cathinone derivatives (butylone, buphedrone, 3,4-dimethylmethcathinone [3,4-DMMC], and 4-fluoromethcathinone [4-FMC or flephedrone]) and benzofurans (6-(2-aminopropyl)benzofuran (6-APB) and 5-(2-aminopropyl)benzofuran) (5-APB).

1. Synthetic cathinones

Synthetic cathinones derive from the naturally occurring alkaloid cathinone, that comes from the evergreen plant *Catha edulis*. They are known by the street name of *bath salts* and have been marketed as legal alternatives to classical stimulants such as amphetamines. The majority of these substances are manufactured in China and South East Asia and then shipped to distributors, who are in charge of packaging, selling and, eventually, tampering (common adulterants include paracetamol, caffeine and piperazines). The packaging often includes the warning '*not for human consumption*', as well as names such as *Ivory Wave*, *Bloom* and *Vanilla Sky* (German et al., 2014; Valente et al., 2014).

1.1. Chemistry

The chemical structure of cathinone is very similar to that of amphetamine, with an additional β-keto group on the amino alkyl chain (Fig. 1).

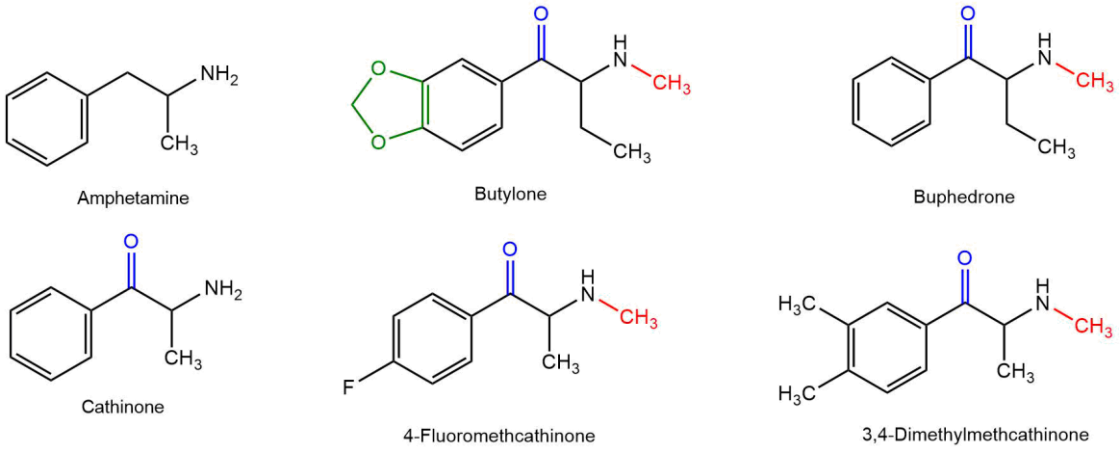


Figure 1 Chemical structure of amphetamine, cathinone and the synthetic cathinones butylone, buphedrone, 4-fluoromethcathinone and 3,4-dimethylmethcathinone

This group is present in all synthetic cathinones, which are synthesised by adding extra groups to the basic cathinone structure (Valente et al., 2014). Aside from the ketone group, synthetic cathinones may have other functional groups, and their presence or absence classifies the compound in one of four families (Fig. 1):

- N-alkylated synthetic cathinones (*e.g.* buphedrone, 3,4-dimethylmethcathinone [3,4-DMMC] and 4-fluoromethcathinone [4-FMC or flephedrone]);
- synthetic cathinones with a 3,4-methylenedioxy ring (*e.g.* butylone);
- N-pyrrolidine substituted synthetic cathinones; and
- cathinone derivatives that present the chemical characteristics of the former two groups, *i.e.* n-pyrrolidine substituted synthetic cathinones with a 3,4-methylenedioxy ring.

The ketone group of synthetic cathinones is responsible for a decrease in polarity, when comparing with its amphetamine counterpart. However, analogues displaying the pyrrolidine ring at the amino group present higher lipophilicity. The increase or decrease in polarity will affect the compounds capability to cross the blood-brain barrier (BBB) and to reach the brain.

1.2. Pharmacodynamics

Upon reaching the brain, cathinone derivatives will interact with the monoamine reuptake transporters, functioning as substrates or blockers and inducing dopamine (DA), serotonin (5-HT) and noradrenaline (NA) release. The distinct capacity that these drugs have for inducing monoamine release and for interacting with monoamine transporters led to the classification of synthetic cathinones in three classes (Table 1) (Valente et al., 2014; Zawilska and Wojcieszak, 2013).

Table 1 Classification of synthetic cathinones according to the pharmacological action. MDMA, 3,4-methylenedioxymethamphetamine. 5-HT, serotonin. DA, dopamine. 4-FMC, 4-fluoromethcathinone

Class	Mode of action
Cocaine-MDMA-mixed cathinones (*e.g.* butylone and buphedrone)	Act as non-selective monoamine reuptake inhibitors and induce 5-HT release
Methamphetamine-like cathinones (*e.g.* 4-FMC)	Induce DA release and inhibit catecholamine reuptake
Pyrovalerone cathinones	Do not provoke monoamine release. Act as weak 5-HT reuptake inhibitors and potent catecholamine reuptake inhibitors

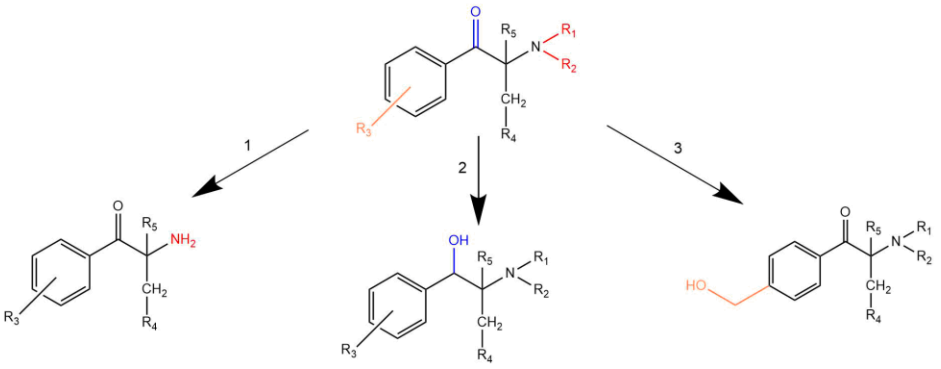
Different synthetic cathinones will display different affinities for monoamine transporters, even if they belong to the same class. Accordingly, 4-FMC is more capable of inhibiting the NA transporters (NAT) than buphedrone or butylone, but butylone has a higher inhibitor potency for the DA and 5-HT transporters (DAT and SERT, respectively) than 4-FMC and buphedrone (Simmler et al., 2013; Simmler et al., 2014). It is also known that cathinone derivatives are capable of inhibiting monoamine oxidase (MAO) and have increased affinity for the isoform MAO-B, the main enzyme responsible for degrading DA. Additionally, the methyl group present at the α-carbon prevents the degradation of these compounds by MAO (Kelly, 2011; Valente et al., 2014).

1.3. Pharmacokinetics

The most common administration routes for synthetic cathinones are oral ingestion and nasal inhalation (‘snorting’), but many other methods can be employed (such as rectal insertion, intramuscular or intravenous injection) (Prosser and Nelson, 2012).

After absorption, the compounds will undergo extensive phase I metabolism (Kelly, 2011). Buphedrone, 3,4-DMMC, and 4-FMC display the common metabolic pathways of N-

alkylated synthetic cathinones, which include N-demethylation and reduction of the ketone group (Fig. 2) (Uralets et al., 2014).



Name	R_1	R_2	R_3	R_4	R_5
Buphedrone	H	CH_3	H	CH_3	H
3,4-DMMC	H	CH_3	H	CH_3	H
4-FMC	H	CH_3	F	CH_3	H

Figure 2 Main metabolic pathways of N-alkylated synthetic cathinones. Reactions are 1 - N-dealkylation, 2 - βketo reduction and 3- ring hydroxylation (except for buphedrone).

A detailed study regarding the metabolism of 3,4-DMMC after administration of approximately 30 mg of the drug showed the production of three main urine metabolites, 3,4-dimethylcathinone (DMC), β-hidroxy-DMMC, β-hidroxy-DMC, and other putative metabolites of unknown structure (Fig. 3). Furthermore, partial conjugation of the parent drug and its metabolites is a possibility, since the concentration of metabolites increased following urine hydrolysis (Tyrkko et al., 2013).

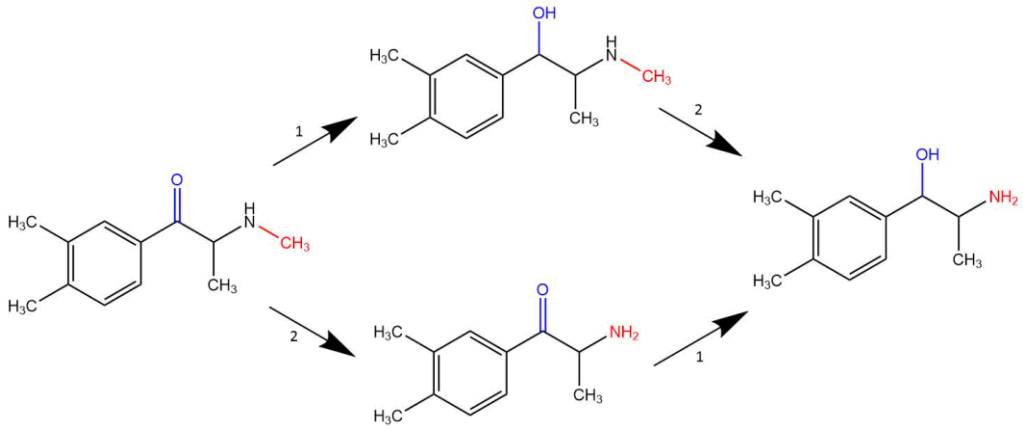


Figure 3 Metabolic pathways and metabolites of 3,4-dimethylmethcathinone (3,4-DMMC). Reactions 1 and 2 are β-keto reduction and N-demethylation, respectively

In what concerns 4-FMC, the drug is expected to be more resistant to metabolism due to the presence of the C-F bond, which increases the molecule stability and therefore the resistance to cleavage (Kelly, 2011). A study in rats regarding the metabolism of the isomer 3-fluoromethcathinone (3-FMC) provided information on the metabolic pathways of the drugs, which include ring hydroxylation, reduction of the ketone group, and N-demethylation (Fig. 4).

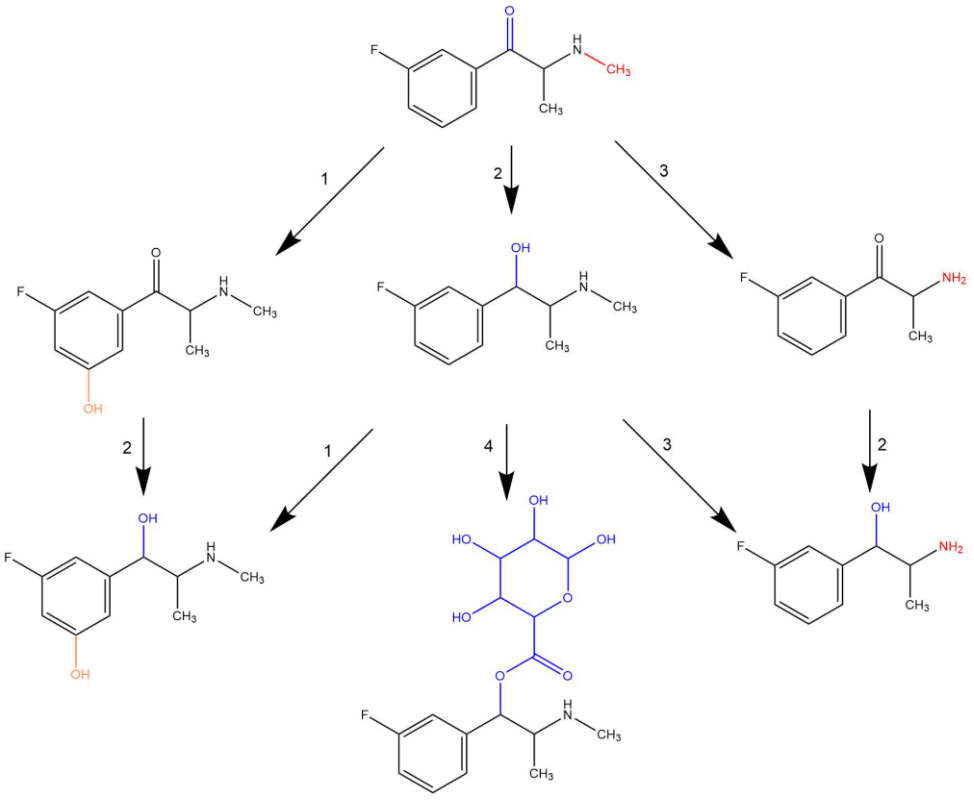


Figure 4 Metabolic pathways of 3-fluoromethcathinone (3-FMC). Reactions are 1- ring hydroxylation, 2- β-keto reduction, 3- N-demethylation, and 4- conjugation with glucuronic acid (adapted from Meyer (2012))

3-FMC can be excreted in the non-metabolized form, after reduction of the ketone group, or as a conjugate with glucuronic acid (Meyer et al., 2012).

Butylone also suffers reduction of the β-keto group and N-dealkylation (the last one appears to be a minor pathway for synthetic cathinones). Additionally, demethylenation of the methylenedioxy ring, followed by O-methylation by catechol O-methyl transferase (COMT) also occurs (Fig.5). The resulting metabolites can be conjugated with sulphate or glucuronide and are excreted with the parent drug (Zaitsu et al., 2011).

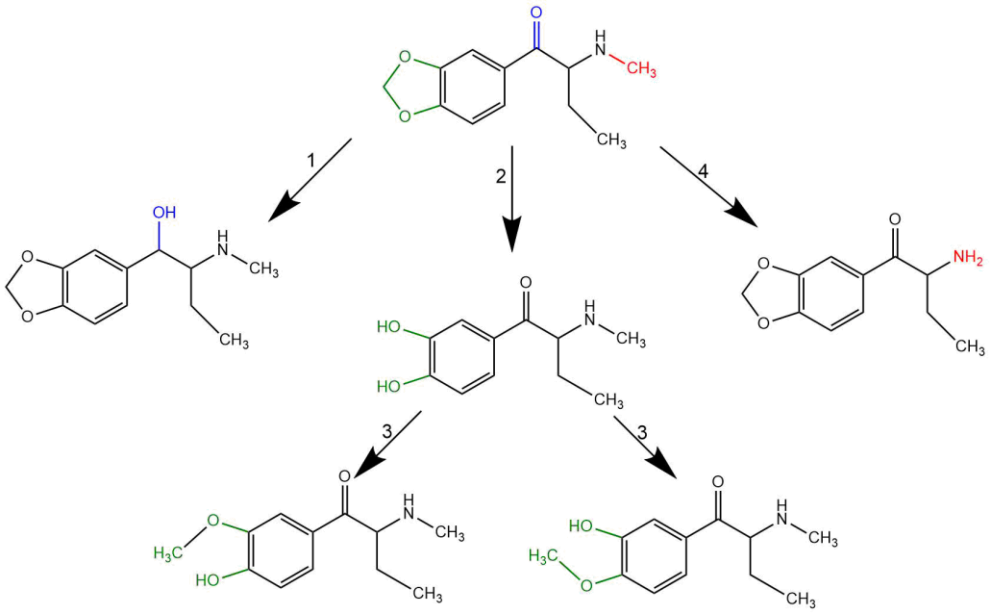


Figure 5 Metabolic pathway of butylone. Reactions; 1 – β-keto reduction, 2 – demethylenation, 3 – O-methylation and 4 – N-dealkylation.

1.4. Subjective effects in human

Among the main pleasant effects of synthetic cathinone, abusers describe euphoria, increased in energy, motivation, and empathy. However, undesired effects will also occur; the most reported are associated with sympathomimetic syndrome and include agitation, hypertension, tachycardia, seizures, mydriasis, and hyperthermia (Coppola and Mondola, 2012; Katz et al., 2014; Kelly, 2011; Prosser and Nelson, 2012; Valente et al., 2014).

1.5. Toxicity

Synthetic cathinones have been documented to cause toxicity at numerous levels. In what concerns neurotoxicity, 3-FMC has been proven capable of inhibiting cellular growth of mouse hippocampal cells (Siedlecka-Kroplewska et al., 2014). Toxicity towards other important organs needs further investigation, but the already existing studies show that cathinone derivatives elicit toxic effects on heart (increased heart rate, blood pressure, and mean arterial pressure in rats), liver (induction of concentration-dependent death in isolated hepatocytes), kidneys (acute kidney injury in abusers), and muscle tissue (rhabdomyolysis); and are capable of inducing hyperthermia, which in its turn may aggravate all the other deleterious effects, as already observed for amphetamines (Araujo et al., 2015; da Silva et al., 2014a; Dias da Silva et al., 2013c; Fantegrossi et al., 2013; Kiyatkin et al., 2015; Meng et al., 2012; O'Connor et al., 2015; Sutamtewagul et al., 2014; Valente et al., 2016; Valente

et al., 2015). The mechanisms through which these compounds trigger toxicity need further investigation, but it was already shown that synthetic cathinones cause an increase in oxidative stress, hamper cellular energetics and Ca^{2+} homeostasis, cause mitochondrial dysfunction and apoptosis (Valente et al., 2016; Valente et al., 2015). It is still necessary and paramount to clarify whether these effects are extensible to all synthetic cathinones and if so, at which levels they negatively affect abusers.

2. Benzofurans

Another popular group of NPS, which are also synthetic phenethylamines, is benzofurans. The most commonly consumed benzofurans are 5-(2-aminopropyl)benzofuran (5-APB) and 6-(2-aminopropyl)benzofuran (6-APB), substances that will also be addressed in this work. These compounds appeared in the drug scene in 2010/2011 and are best known under the name '*benzo fury*' (Zawilska, 2015). They were first synthesized in 1993, as non-neurotoxic analogues of methylenedioxymethamphetamine (MDMA).

2.1. Chemistry

Structurally, benzofurans are closely related to the MDMA active metabolite methylenedioxyamphetamine (MDA), with a furan instead of a methylenedioxy ring (Fig. 6) (Jebadurai et al., 2013; Nugteren-van Lonkhuyzen et al., 2015).

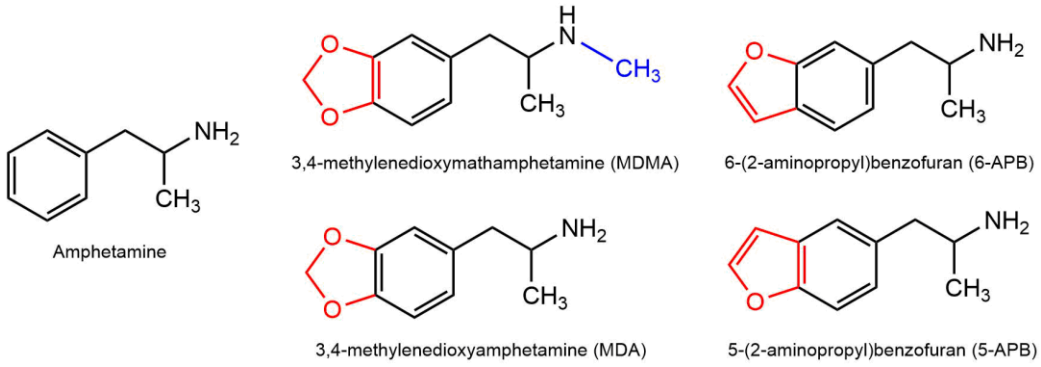


Figure 6 Chemical structure of benzofurans and of their amphetamine analogues.

2.2. Patterns of abuse

While 5-APB misuse started in United Kingdom in 2010, first 6-APB reports occurred in Hungary in the subsequent year. The trading of these drugs rapidly increased through

internet, in particular for 6-APB (EMCDDA, 2011). The most common route of administration is oral ingestion; inhalation is described as very painful (Jebadurai et al., 2013).

2.3. Pharmacodynamics

In a similar fashion to MDMA, 6-APB and 5-APB act primarily as potent monoamine transporter inhibitors, and are also capable of inducing monoamine release and of interacting with the trace amine-associated receptor 1 (TAAR1), which is thought to underline locomotion and the neurochemical stimulant effects of these drugs (Rickli et al., 2015). Additionally, both benzofurans demonstrated to be partial agonists of the serotoninergic receptors 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}. This binding is particularly important since 5-HT_{2A} is associated with the hallucinogenic properties of the drugs and 5-HT_{2B} stimulation can increase the risk of cardiotoxicity by inducing proliferation of cardiac cells of the interstitial valve, thus leading to drug-induced heart valve fibrosis (Iversen et al., 2013; Rickli et al., 2015). The analogues 6-APB and 5-APB were also capable of binding to α-adrenoreceptors; of particular interest, the high affinity of 6-APB to α_{2A} receptor, is responsible by intense release of NA and, consequently, sympathomimetic effects (Rickli et al., 2015). In addition, 5-APB proved to be capable of inducing vasoconstriction, an effect mediated by 5-HT_{2A} receptors, and associated to increased DA brain levels (Dawson et al., 2014).

2.4. Pharmacokinetics

The main metabolites of 6-APB and 5-APB are 4-carboxymethyl-3-hydroxy amphetamine and 3-carboxymethyl-4-hydroxy amphetamine, respectively. The main metabolic pathways involved include ring cleavage by hydroxylation and reduction of the resulting unsaturated aldehyde, which can then be oxidized to a carboxylic acid or reduced to an alcohol. Some minor metabolic steps include deamination of both 6-APB and 5-APB and di-hydroxylation of 5-APB (Fig. 7) (Welter-Luedeke and Maurer, 2015).

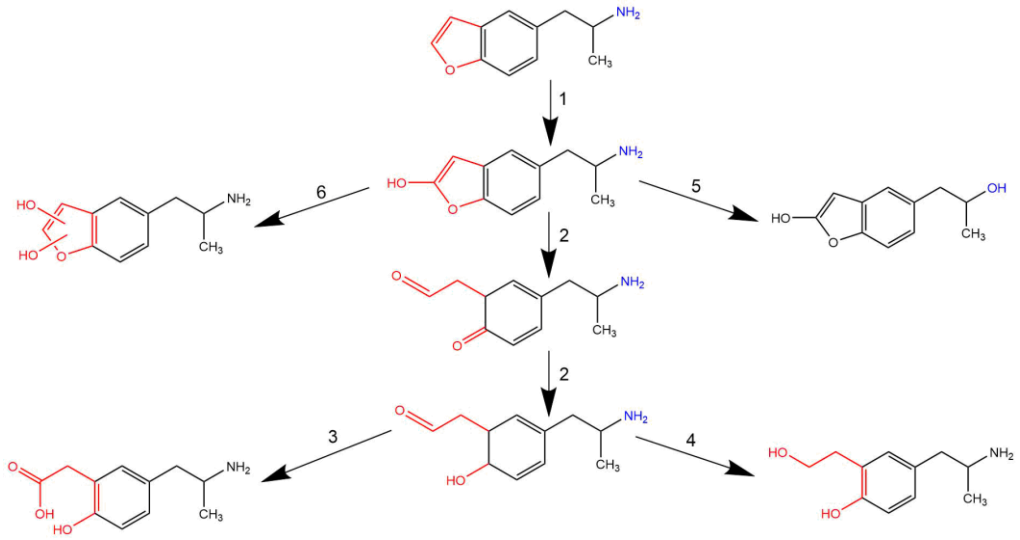


Figure 7 Metabolic pathways of 5-APB. Reactions: 1 - ring hydroxylation, 2 - reduction of unsaturated aldehyde, 3 - oxidation to carboxylic acid, 4 - reduction to alcohol, 5 - deamination and 6 - further hydroxylation of the hidroxy metabolite

2.5. Subjective effects in humans

Benzofurans are ingested mostly due to their ability to induce entactogenic and stimulant effects, such as MDMA but with higher potency (Zawilska, 2015).The adverse effects caused by these substances are in league those elicited by MDMA and MDA, with users reporting tachycardia, agitation, nausea, anxiety, jaw and teeth clenching, and mydriasis (Jebadurai et al., 2013; Zawilska, 2015).

2.6. Toxicity

Of concern, 5-APB and 6-APB have been implicated in a number of deaths in the UK, but in the majority of the cases there were other substances present (Nugteren-van Lonkhuyzen et al., 2015). A recent study evaluated the cytotoxic potential of 5-APB in isolated rat hepatocytes and concluded that this substance has the ability to induce time-dependent cytotoxicity, to increase cellular oxidative stress, and to interfere with mitochondrial homeostasis (Nakagawa et al., 2016). It is clear that benzofurans have the potential to cause severe toxicity, but the mechanisms involved are yet to be fully unravelled. Therefore, it is paramount for user safety the conduction of more studies pertaining to benzofurans toxicity.

Aim

Research on the toxicity of NPS is imperative because the vast majority of these substances are marketed without testing their safety prior to human consumption. Synthetic phenethylamines comprise a great part of the new substances that reach the drug markets, and every time a substance becomes prohibited, new derivatives are synthesized to fill its gap. With the intense rate of production of NPS, it is difficult to accompany their appearance in the drug scene with satisfactory knowledge on the mechanisms of toxicity. However, in virtue of the structural similarity between banned and newly synthesised drugs, information on toxicity of the previously legal drugs is a useful tool.

In this context, the synthetic cathinones butylone, buphedrone, 3,4-DMMC, and 4-FMC; and the benzofurans 6-APB and 5-APB are NPS that lack information on the mechanisms underlying the toxicity reported in clinical cases. Notwithstanding, these drugs are still available and consumed. Since oral ingestion is one of the major administration routes for this type of stimulants, it is natural that liver represents one of the main targets of NPS toxicity, largely due to the first passage effect. Also, due to the important role of liver in metabolism, this phenomenon may implicate the generation of more toxic metabolites that may exert cytotoxic effects at this level, compromising the good functioning of the organ and, therefore, the overall user health.

Bearing all of this in mind, the aims of the present work were i) to determine the hepatotoxic potential of butylone, buphedrone, 3,4-DMMC, 4-FMC, 5-APB, and 6-APB; and ii) to clarify the toxicological mechanisms by which the observed effects occur.

Materials and Methods

1. Chemicals and reagents

Butylone, buphedrone, 6-APB, 5-APB, 3,4-DMMC, and 4-FMC were acquired online at sensearomatics.com on April 2013. All drugs were used as provided, in powder form, except for 6-APB, which came in pill form and required pulverization before use. Stock solutions of all the drugs were made up in Hanks' balanced salt solution (HBSS, no calcium or magnesium) and stored at -20º C. These solutions were thawed and diluted on the days that experiments were performed.

Cell culture reagents were purchased from GIBCO Invitrogen (Alfagene, Lisboa, Portugal) and all other reagents were obtained from Sigma-Aldrich (Lisboa, Portugal), unless otherwise stated.

2. Isolation and culture of primary rat hepatocytes

Primary rat hepatocytes were isolated from male Wistar Han rats weighing between 200-250 g, purchased from Charles River (Barcelona, Spain). The surgical procedures approved by the local ethics committee for the welfare of experimental animals were conducted according to national legislation between 10.00 and 11.00 am. The animals were anesthetized with isoflurane (IsoFlo®, Abbot Laboratories, Berkshire, UK) and cells were isolated through collagenase perfusion method, as previously described by Moldeus (1978). Briefly, following the insertion of a cannula in the portal hepatic vein, the liver was perfused with an oxygenated Hank's washing buffer containing the chelator ethylene glycol tetraacetic acid (EGTA). After this initial wash, the extracellular concentration of calcium decreased significantly, resulting in an irreversible cleavage of the desmosomes (junctional complexes). Then, the liver was perfused with a solution of collagenase supplemented with its cofactor calcium. The hepatic capsule was then disrupted gently, in order to release the isolated cells into a Krebs-Henseleit buffer. This cellular suspension was purified by 3 low-speed centrifugation cycles (50 x g, 2 minutes, 4º C) and then incubated with penicillin/streptomycin (500 U/ml; 500 μg/ml) for 30 minutes at 4ºC. Cell viability was then estimated by the trypan blue exclusion test, with results always above 85%. The suspension with a cellular density of 5 $x10^5$ was cultured in Williams' E medium (Sigma-Aldrich) supplemented with 10% heat-inactivated foetal bovine serum (FBS), penicillin/streptomycin (100 U/ml; 100 μg/ml), 50 μg/ml dexamethasone (Sigma-Aldrich), 5 μg/ml insulin (Sigma-Aldrich), 2,5 μg/ml fungizone, and 100 μg/ml gentamicin. Cells were seeded onto 96-well plates, at a density of 50,000 per well in a volume of 100 μL, or 6-well plates at a density of

5,000,000 per well in a volume of 1 mL and left to adhere overnight, at 37 ºC with a 5% CO_2 atmosphere.

3. Immortalized cell lines

Two different hepatic cell lines were used for testing the toxic potential of our compounds, HepaRG and HepG2. HepaRG is a cell line derived from a female patient with hepatocarcinoma. Cells should be seeded at a low density; upon reaching confluence they can transdifferentiate into two types of cells, one that is morphologically similar to human primary hepatocytes, and another that resembles bile canaliculus-like structures (Guillouzo et al., 2007). Another particularity of the HepaRG cell line is that it retains the ability to express all major cytochrome P450 (CYP), with exception of CYP2E1 and CYP2D6, and many phase II enzymes, mimicking closely the *in vivo* situation. HepG2 cells were isolated from a male patient with hepatoblastoma, and also express normal hepatocyte function. In addition, these cells secrete into the culture medium the majority of plasma proteins and retain the capacity to produce and secrete bile acids (Bouma et al., 1989).

HepaRG cells were acquired from Life Technologies (Invitrogen, France) and were cultured in 75 cm^2 flasks using Williams' E medium with L-glutamine supplemented with 10% FBS, penicillin/streptomycin (10000 U/ml; 10000μg/ml), 5 μg/ml insulin (Sigma-Aldrich), and 50 μM hydrocortisone 21-hemisuccinate sodium salt (Sigma-Aldrich). The environmental conditions were 37 ºC with an atmosphere with 5% CO_2; the medium was changed every 2-3 days, allowing the cells to grow. Cells were sub-cultured for a maximum of 10 passages by tripsinization, and allowed to reach 80% confluence. Upon reaching confluence, the differentiation process was started by changing the media to fresh culture media supplemented with 2% DMSO (Merck); the medium was changed every two days, for two weeks, in order to promote a ratio of hepatic cells 1: biliary cells 1. At the end of this time, the cells were seeded into 96-well plates at a density of 144,000 cells per well in a volume of 200 μL. Cells were left to adhere overnight and were used for cytotoxicity assays on the following day.

HepG2 cells were provided by Prof. Ricardo Dinis-Oliveira (Department of Sciences, Advanced Institute of Health Sciences-North, CESPU, CRL, Gandra, Portugal) and cultured using DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin (10000 U/ml; 10000μg/ml), in 75 cm^2 flasks. The cells were kept in a humidified 5% CO_2 atmosphere at 37ºC and subjected to regular medium changes (every two-three days). Whenever cells reached 80% confluence, they were sub-cultured for no more than 10

passages by tripsinization. For the cytotoxicity assays, cells were seeded at a density of 80,000 cells per well, in a volume of 100 μL, in 96-well plates, and left to adhere overnight.

4. Viability assay

The cytotoxicity of the chosen compounds was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, which measures cell viability indirectly through succinate dehydrogenase activity, an indicator of healthy and functional mitochondria. On the day of the experiment, the cells were exposed to a vast range of concentrations of all drugs, in triplicates. In each plate, positive (1% Triton X-100) and negative controls (culture medium) were also included. The tested concentration range for each drug across the different cell models is displayed in Table 2. The MTT results were used to calculate the concentrations of each drug to be tested in the further assays. Accordingly, the concentrations of each drug producing 20%, 40%, 50% and 70% of the maximum effect in the MTT assay (EC_{20}, EC_{40}, EC_{50} and EC_{70}, respectively) were estimated from the respective concentration *versus* response curves, using the parameters of the best fit regression model.

Table 2 Concentration range (mM) tested for each substance, in the different cell models

	Butylone	Buphedrone	6-APB	5-APB	3,4-DMMC	4-FMC
Primary rat hepatocytes	0.03770 – 10.0000	0.03770 – 10.0000	0.03770 – 23.0000	0.03770 – 10.0000	0.0011 – 0.7000	0.03770 – 10.0000
HepaRG cells	0.009600 – 18.1500	0.4900 – 32.0000	1.1706 – 14.0000	0.1100 – 6.5000	0.08780 – 2.0000	1.4632 – 30.0000
HepG2 cells	1.5937 – 20.0000	0.6252 – 30.0000	0.1924 – 30.0000	0.2926 – 20.0000	0.05840 – 2.0000	0.5861 – 30.0000

5. Total (tGSH), reduced (redGSH), and oxidized (GSSG) glutathione

Samples preparation: Primary rat hepatocytes seeded in 6-well plates were exposed to the EC_{20}, EC_{40}, EC_{50}, and EC_{70} of each testing drug, for a period of 24h, at 37 ºC. After this time, the cells were washed with HBSS with calcium and magnesium, and then precipitated using 5% perchloric acid for 20 minutes, at 4 ºC. Cells were scrapped and the obtained suspension was centrifuged at 13,000 *g* for 2 minutes, at 4 ºC. The supernatant was collected and stored at -80 ºC until further determinations. The cell pellet was resuspended in 1M NaOH and used to quantify protein.

The method used to measure tGSH was the 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB)-GSSG reductase-recycling assay(da Silva et al., 2014a). After thawing, the supernatants were neutralized using 0.76 M $KHCO_3$ and centrifuged at 13,000 *g* for 10 minutes, at 4 ºC. Then, 100 μL of each supernatant, blanks and standards were transferred to a 96-well plate, followed by the addition of 65 μL reagent solution (72 mM phosphate buffer, 0.69 mM NADPH, and 4 mM DTNB). The plates were incubated for 15 minutes, at 30 ºC, in a multi-well plate reader (Power Wave XTM, BioTek Instruments, Inc.) and then 40 μL of a 10 U/mL glutathione reductase solution was added. The absorbance of the samples were further read in kinetic mode at 415 nm in order to follow the formation of 5-thio-2-nitrobenzoic acid (TNB). The sample results were compared with a standard curve, performed in every experiment. The same protocol was used for the quantification of GSSG, with the additional step of incubating the samples with 2-vinylpyridine for 1 hour, at 4 ºC. Experimental data were normalized to the protein content of each sample. The intracellular redGSH was calculated using the formula redGSH = tGSH – 2 x GSSG.

6. Intracellular adenosine triphosphate (ATP)

Samples were prepared as described before in '5. Total (tGSH), reduced (redGSH), and oxidized (GSSG) glutathione'. A bioluminescence method was used for measuring intracellular ATP, as previously described. After the neutralization with $KHCO_3$, the samples were centrifuged at 13,000 *g* for 3 minutes, at 4 ºC. Afterwards, 75 μL of each supernatant, standard, or blank was transferred into a white 96-well plate. Then, 75 μL of a luciferin-luciferase solution (0.15 mM luciferin; 30,000 light units luciferase; 10 mM $MgSO_4$; 50 mM glycine;1 mM Tris; 0.55 mM EDTA; 1% BSA) were added and the emitting light intensity was measured using a luminescence plate reader (BioTek Instruments, Vermont, USA). The results were compared to a standard curve performed in every experiment, and normalized to the protein content.

7. Protein determination

Protein quantification of samples was performed using the Lowry assay, as established previously (Lowry et al., 1951).

8. Intracellular reactive oxygen (ROS) and nitrogen (RNS) species

The assessment of intracellular ROS and RNS was performed using the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence assay, as described by da Silva (2014a). Briefly, primary rat hepatocytes seeded in 96-well plates were exposed for 30 minutes to 10 μM DCFH-DA, at 37 ºC, protected from light. Afterwards, the cells were

washed using HBSS and incubated with the EC_{20}, EC_{40}, EC_{50} and EC_{70} of each tested drug, at 37 ºC, for 24 hours. The fluorescence was recorded using a fluorescence microplate reader (BioTek Instruments, Vermont, USA), set to 485 nm excitation and 530 nm emission. The acquired data was normalized to control conditions (no drug exposure).

9. Statistical Analysis

Data obtained in the MTT assay in four independent experiments were fitted to the Logit model, chosen based on the goodness-to-fit principle (best fit dosimetric model). The results of three different experiments obtained for tGHS, GSSG, ATP, and ROS/RNS determinations are presented as mean ± standard error of the mean (SEM). The normality of data distribution was analysed using the Kolmogorov-Smirnov test and statistical comparison between groups was performed either: i) using one-way analysis of variance (ANOVA), followed by Dunn's multiple comparison test when the results followed a normal distribution; or ii) using Kruskal-Wallis test, when the data did not follow a normal distribution. All statistical analysis were made using GraphPad Prism software, version 6.07 (GraphPad Software, San Diego, CA, USA).

Results

1. Synthetic cathinones and benzofurans elicited *in vitro* hepatotoxicity in a concentration-dependent manner

The cytotoxic effects observed *in vitro* for all the tested compounds in three different hepatocyte models are presented in Figure 8.

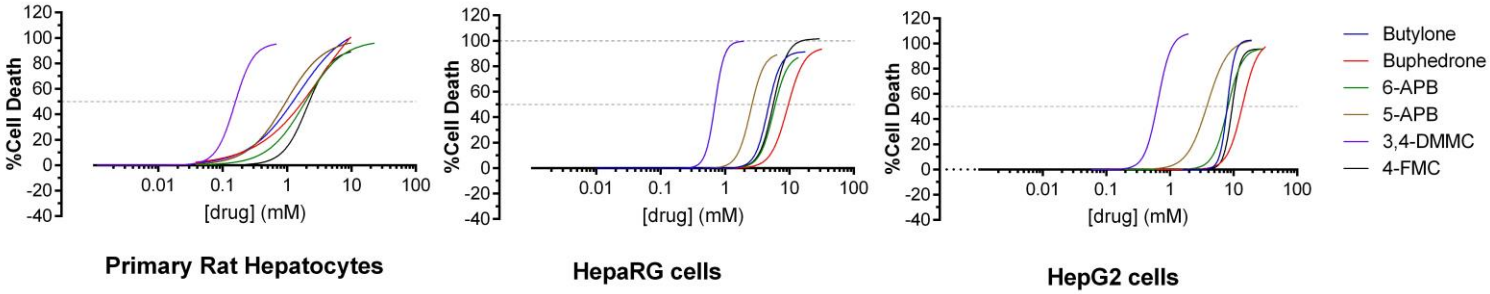


Figure 8 Concentration-response curves for butylone, buphedrone, 6-(2-aminopropyl)benzofuran (6-APB), 5-(2-aminopropyl)benzofuran (5-APB), 3,4-dimethylmethcathinone (3,4-DMMC), and 4-fluoromethcathinone (4-FMC) in primary rat hepatocytes, HepaRG and HepG2 cells, following 24 h incubation at 37 ºC. Cell mortality was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay and data were normalized to positive (100%) and negative (0%) controls (n=4).

Our data show that the drug-induced cell death is concentration-dependent. The EC_{50} values of each compound, for each *in vitro* model, extrapolated from the regression model that best suited the respective data set (*i.e.* Logit model) are presented in Table 3. The most toxic drug for all the evaluated models was 3,4-DMMC, which elicited 50% response at the lowest concentrations, when compared with the other drugs. Contrarily, 4-FMC proved to be the least toxic drug for primary rat hepatocytes, while for the cell lines HepaRG and HepG2, buphedrone presented the highest EC_{50} values. By analysing the concentration-response curves and comparing effect concentrations, it is clear that primary rat hepatocytes display an increased sensitivity for hepatotoxicity, while HepaRG and HepG2 cells are more resistant to the toxic effects of these drugs. Therefore, further assays were carried out on this model.

Table 3 EC_{50} values (mM) for the cytotoxicity of butylone, buphedrone, 6-(2-aminopropyl)benzofuran (6-APB), 5-(2-aminopropyl)benzofuran (5-APB), 3,4-dimethylmethcathinone (3,4-DMMC), and 4-fluoromethcathinone (4-FMC), obtained in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, following incubation of each hepatocyte model for 24 h, at 37 ºC

	Butylone	**Buphedrone**	**6-APB**	**5-APB**	**3,4-DMMC**	**4-FMC**
Rat hepatocytes	1.2110	1.5702	1.9360	0.9636	0.1578	2.2127
HepaRG	4.8073	9.6185	6.0204	2.6170	0.6953	5.5866
HepG2	7.9213	13.672	8.1816	3.7885	0.6223	9.5679

After media aspiration, following primary hepatocyte drug exposures, we verified the formation of intracellular black-brown pigments, at the highest concentrations tested. This observation occurred before addition of the MTT.

2. Benzofurans and synthetic cathinones induce oxidative stress in primary rat hepatocytes

Induction of oxidative stress is one of the cornerstone toxicity mechanisms of classic stimulants, such as amphetamines (Carvalho et al., 2012). Given the verified capacity of butylone, buphedrone, 6-APB, 5-APB, 3,4-DMMC, and 4-FMC to induce hepatotoxicity, the next step was to determine the role of oxidative stress in this process, by evaluating the formation of ROS and RNS in rat hepatocytes 24 h after exposure to the drugs. In addition, since GSH is a pivotal first-defence against free radicals, the intracellular levels of GSH and GSSG were also measured. For these assays, primary rat hepatocytes were exposed to concentrations that elicited mortality of 20%, 40%, 50%, and 70%, as calculated based on the MTT assay results. The corresponding drug concentrations are presented in Table 4.

Table 4 Effect concentrations (ECx) of butylone, buphedrone, 6-(2-aminopropyl)benzofuran (6-APB), 5-(2-aminopropyl)benzofuran (5-APB), 3,4-dimethylmethcathinone (3,4-DMMC), and 4-fluoromethcathinone (4-FMC) that elicit x% of effect (i.e. mortality) in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, following incubation of primary hepatocytes for 24 h, at 37 ºC

x (%)	Effect concentration (ECx) for the drug with the effectiveness x (mM)					
	Butylone	**Buphedrone**	**6-APB**	**5-APB**	**3,4-DMMC**	**4-FMC**
70	2.669	4.151	4.575	2.151	0.232	3.958
50	1.381	2.831	2.737	1.332	0.167	2.859
40	0.982	2.343	2.046	1.051	0.142	2.454
20	0.415	1.469	0.933	0.587	0.096	1.701

Figure 9 depicts the results obtained in the DCFH-DA assay utilised for evaluating the production of ROS and RNS, after an incubation period of 24h. As observed, 4-FMC elicited the greatest production of ROS and RNS. When the cells were exposed to the drug concentrations that elicited 20%, 40%, 50%, and 70% of response in the MTT assay, the observed results were significantly different from control ($p < 0.05$ for EC_{20}, $p < 0.0001$ for EC_{40} and EC_{50}, and $p < 0.001$ for EC_{70}; Figure 8). Also, 3,4-DMMC caused a significant increase in ROS and RNS production, which in this case was dependent on concentration ($p < 0.01$ for EC_{40}, $p < 0.001$ for EC_{50}, and $p < 0.0001$ for EC_{70}). Butylone at the

concentration EC_{70} also caused a significant increase in ROS and RNS ($p < 0.001$), as it did the EC_{40} and EC_{70} of buphedrone ($p < 0.05$).

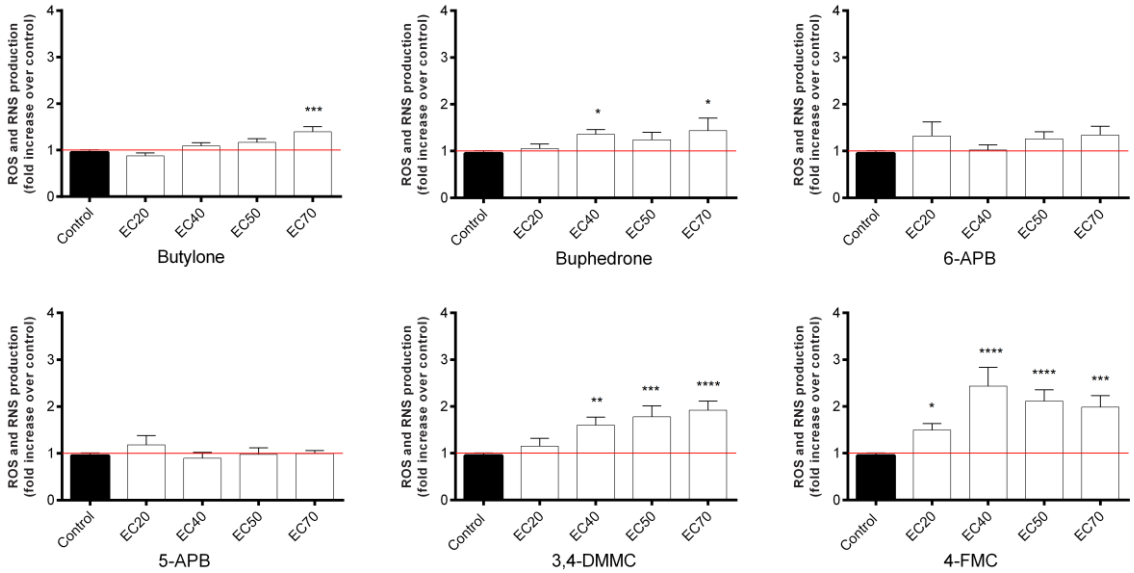


Figure 9 Production of reactive oxygen and nitrogen species (ROS and RNS, respectively) in primary rat hepatocytes, after exposure to the EC_{20}, EC_{40}, EC_{50}, and EC_{70} of butylone, buphedrone, 6-(2-aminopropyl)benzofuran (6-APB), 5-(2-aminopropyl)benzofuran (5-APB), 3,4-dimethylmethcathinone (3,4-DMMC), and 4-fluoromethcathinone (4-FMC) for 24h, at 37ºC. Fluorescence data from the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) assay were normalised to negative controls (set to 1) and were from four independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; compares to control. Statistical analysis of data was performed using one-way ANOVA and Dunn's multiple comparison test.

The results for the quantification of GSH and GSSG are presented in Figure 10.

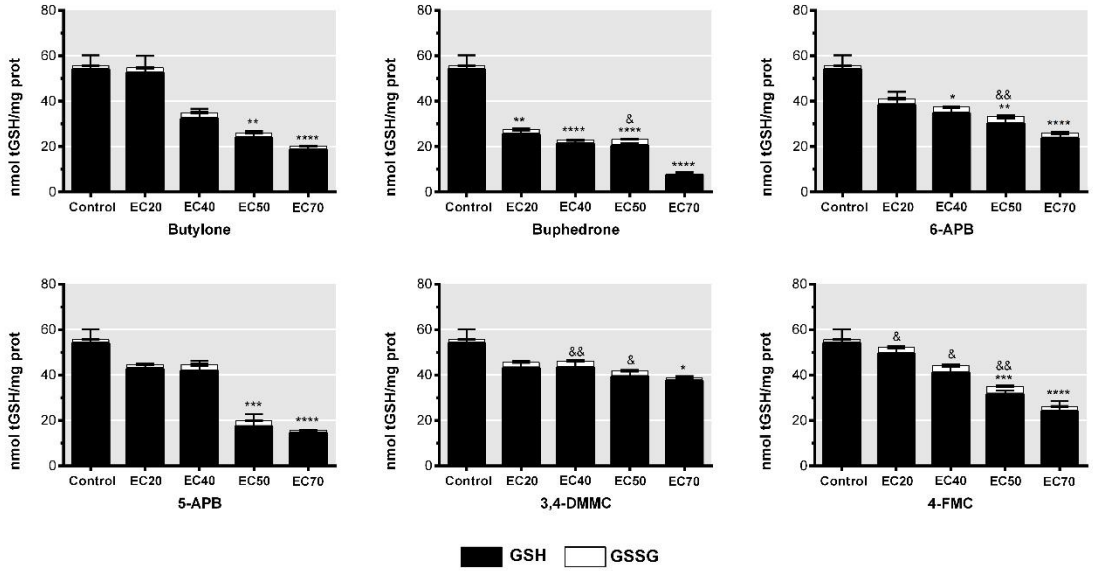


Figure 10 Intracellular levels of total (tGSH), reduced (GSH) and oxidized (GSSG) glutathione in primary rat hepatocytes following 24h incubation with butylone, buphedrone, 6-(2-aminopropyl)benzofuran (6-APB), 5-(2-aminopropyl)benzofuran (5-APB), 3,4-dimethylmethcathinone (3,4-DMMC), and 4-fluoromethcathinone (4-FMC), at 37 ºC. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; compares to GSH control; & p<0.05, && p<0.01; compares to GSSG control. Statistical analysis of data was performed using Kruskal-Wallis test.

Butylone, 5-APB, and 4-FMC, tested at the respective EC_{50} and EC_{70} values, caused a very significant ($p < 0.01$ and $p < 0.0001$, respectively) depletion of GSH when compared to control. Buphedrone elicited the most significant thiol disturbance, with EC_{20} showing the least significant GSH decrease ($p < 0.01$), when compared with all other tested EC_x ($p < 0.0001$). At the EC_{50}, buphedrone also significantly increased GSSG levels ($p < 0.05$); while 4-FMC significantly diminished GSSG levels for all tested concentrations, except EC_{70} ($p < 0.05$). All concentrations of 6-APB, except the EC_{20}, elicited a concentration-dependent depletion of GSH; similar to buphedrone, the drug also caused a significant depletion of GSSG ($p < 0.01$), at the EC_{50}. 3,4-DMMC only affected GSH levels at EC_{70} ($p < 0.05$), but significantly increased GSSG levels both at EC_{40} and at EC_{50} ($p < 0.01$ and $p < 0.05$, respectively).

3. Synthetic cathinones and benzofurans cause depletion of intracellular ATP

The ATP is essential for cells to successfully carry out their energy-dependent functions and, consequently, to survive. The incubation of primary rat hepatocytes with the EC_{20}, EC_{40}, EC_{50}, and EC_{70} of each NPS for 24h caused an impairment of cell energetics, as depicted in Figure 11.

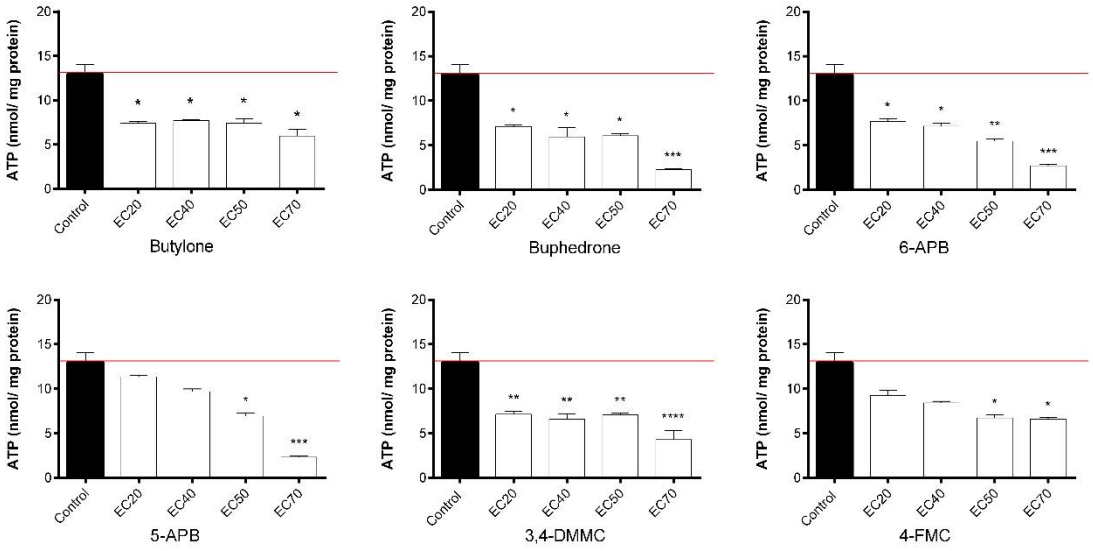


Figure 11 Intracellular adenosine triphosphate (ATP) levels of primary rat hepatocytes following 24h incubation with butylone, buphedrone, 6-(2-aminopropyl)benzofuran (6-APB), 5-(2-aminopropyl)benzofuran (5-APB), 3,4-dimethylmethcathinone (3,4-DMMC) and 4-fluoromethcathinone (4-FMC), at 37 ºC. * (p<0.05), ** (p<0.01), *** (p<0.001), **** (p<0.0001) compares to control. Statistical analysis of data was performed using one-way ANOVA and Dunn's multiple comparison test.

In general, all drugs concentration-dependently decreased the levels of intracellular ATP. These changes were significant for all the concentrations tested, except in the cases of 4-FMC and 5-APB for which toxicity was only significant when tested at EC_{50} and EC_{70} ($p < 0.05$). For buphedrone, 6-APB, 5-APB, and 3,4-DMMC a notorious ATP depletion was

elicited when drugs were tested at EC_{70} ($p < 0.001$ for buphedrone, 6-APB and 5-APB and $p < 0.0001$ for 3,4-DMMC).

Discussion

This work provides the first evidence of hepatotoxicity by the selected synthetic drugs. Previous studies have shed some light upon the toxic potential of some other cathinone derivatives (Araujo et al., 2015; Valente et al., 2016) and of benzofurans (Nakagawa et al., 2016). Butylone, buphedrone, 3,4-DMMC, 4-FMC, and the benzofurans 5- and 6-APB are substances of abuse that have stimulant effects similar to those of amphetamines and, based on the results we presented herein, display similar toxicity mechanisms (Carvalho et al., 2012). To the date we started this work, these mechanisms were barely studied, with little to no information regarding the toxicological potential of these NPS towards such an important organ as the liver.

We performed cytotoxicity studies in three *in vitro* models for the hepatocyte. Our results provided some clues on how these drugs work. HepG2 cells were previously used to successfully demonstrate the hepatotoxicity of other stimulants, namely amphetamines (da Silva et al., 2014a; da Silva et al., 2014b; Dias da Silva et al., 2013a; Dias da Silva et al., 2013b). It is, however, a cell line that expresses very low levels of essential drug-metabolizing enzymes, such as CYP isoforms (Gerets et al., 2012). The use of HepaRG cells – another human hepatoma cell line that display an enzymatic profile more representative of the *in vivo* hepatocyte than HepG2 cells – was therefore of extreme relevance since metabolism may severely impact the toxicity of xenobiotics (Gerets et al., 2012; Rodrigues et al., 2013). In our work, significant differences were verified between the concentration-response curves of the tested drugs in both cell lines. The curves obtained with HepG2 cells were shifted to the right when compared to those obtained with HepaRG cells, and the EC_{50} values obtained for HepG2 were higher than those for HepaRG cells. These findings indicate that HepG2 cells were more resistant to synthetic cathinone and benzofuran toxicity than HepaRG. Altogether, the increased sensitivity shown by these cells and the expression of a more complete enzymatic profile in HepaRG, may indicate that the tested NPS are bioactivated into reactive metabolites or, at least, that metabolism increases hepatotoxicity (e.g., through the reactive species formed from e.g. NADPH oxidase activity). If this prove to be truth, CYP2B6, which has been described to be involved in the metabolism of MDMA and of the isomer 3-FMC (Carvalho et al., 2012; Meyer et al., 2012) may contribute to the observed effects since its levels in HepaRG are very considerable, and surpass those for HepG2 (Aninat et al., 2006). It is possible that other isoforms also have some preponderance, such as CYP1A2 and 3A4, which participate in amphetamine-derivatives metabolism and have greater expression in HepaRG, compared to HepG2 (Aninat et al., 2006; Gerets et al., 2012), yet no studies to clarify the involvement of these isoforms in metabolism of synthetic cathinones and benzofurans have been conducted.

The exception to the observed toxicological differences in both cell lines was 3,4-DMMC; for this drug, the EC_{50} value obtained for HepaRG was slightly higher. Since HepaRG is a cell line established from a donor that poorly expressed CYP2D6 and CYP2E1 (Aninat et al., 2006), it is conceivable that one or both isoforms are involved in the metabolism of the drug. The use of primary rat hepatocytes for evaluating the toxicity of the testing drugs was therefore considered essential to address the flaws presented by the employed cell lines.

The lowest EC_{50} values were attained for all tested NPS in this *in vitro* model, indicating higher susceptibility to the toxicity induced by the drugs. For 3,4-DMMC the EC50 in primary cells was approximately 4x lower, compared to the remaining models, supporting the potential relevance of the absent isoforms to the metabolism of the drug. Accordingly, studies performed in primary rat hepatocytes, revealed that incubation with quinidine, a known CYP2D6 inhibitor, decreased the hepatotoxic potential of some synthetic cathinones (Valente et al., 2016), also corroborating our assumption. This CYP isoform has also demonstrated to play a pivotal role in the metabolism of designer stimulants, such as amphetamines (Carvalho et al., 2012; Pedersen et al., 2013a; Pedersen et al., 2013b), among other drugs of abuse (Dias-da-Silva et al., 2015).

Other causes should be considered in the differential toxic profile of these drugs across the tested cell models, for instance the interaction with nuclear receptors that modulate the expression of CYP isoenzymes, such as the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR); these transcription factors have been found upregulated in HepaRG cells, when compared to HepG2 cells (Aninat et al., 2006), which could impact enzyme activity in this particular model. Another factor that should be considered in the interpretation of these results are membrane transporters (influx/ efflux pumps). It is not yet known which transporters intervene in amphetamines, synthetic cathinones, or benzofurans uptake into hepatocytes and/or the drug efflux. Similarly to what happens in the brain, the organic cation transporter 1 (OCT1) and the multidrug resistance protein 1 (MDR1), which are involved in the influx and efflux of 5-HT and DA (Faber et al., 2003), might also be responsible for transporting these substances. It has been shown that HepG2, HepaRG and primary hepatocytes possess different levels of these transporters (Le Vee et al., 2006). Primary hepatocytes were the cells that expressed the higher and the lower levels of OCT1 and MDR1, respectively; HepG2 presented the lower expression of OCT1; while HepaRG expressed the higher levels of MDR1 (Bachour-El Azzi et al., 2015; Le Vee et al., 2013). Therefore, it is plausible that this differential expression of efflux transporters has an influence in the present results. In order to test this hypothesis, experiments employing specific transporter inhibitors should be carried out in our models to and established the comparisons to the observed cytotoxicity profiles.

As denoted above, the present results suggest that the most significant differences concerning hepatotoxicity among the employed models rely on the activity/content of their CYP isoforms, as these toxic effects appear to be magnified with the increased expression/ activity of the drug metabolizing enzymes. This might signify that the hepatotoxic effects may be due not only to the compounds in itself, but also to their indirect action through toxic metabolites. To verify this hypothesis, further experiments should be conducted either i) by employing specific enzymatic inhibitors with subsequent evaluation of the impact on the toxicity and metabolic profile of butylone, buphedrone, 3,4-DMMC, 4-FMC, 5-APB and 6-APB; or ii) by directly establishing the toxicological profile of the metabolites of these drugs in the same cell models (further comparing the attained results to those obtained for the parent drugs).

It is also possible that interspecies differences account for the discrepancy on the attained EC_{50} values towards the different models, since both cell lines have human origin, but primary hepatocytes derive from rat. It has been proven that rat and human hepatocytes present different metabolic rates of phase I reactions for amphetamines (rat metabolism is faster, compared to humans) and differ in the main metabolic pathways (Green et al., 1986). It should also be pondered that there are specific CYP isoforms for rodents and humans (Astashkina et al., 2012).

Also, it should be considered that the immortalization process to which cell lines are surrendered, endows cells with distinct characteristics, especially regarding the capacity of growth and the extensive lifespan; as the expression profile of several genes has been shown to vary between passages, different physiological responses may arise as a consequence of aberrant expression of intracellular proteins, loss of polarity, and significant decline/absence of important morphological features, such as transporters (Astashkina et al., 2012). This can result in data of difficult interpretation across different passages and among laboratories.

One important finding of in our work, occurred during the establishment of the concentration-response curves in primary rat hepatocytes; we verified, for the highest concentrations tested, the formation of intracellular brown-black pigments. A similar occurrence had already been observed for amphetamines and it was attributed to the formation of aminochromes (da Silva et al., 2014b). The formation of these aminochromes involves metabolism of amphetamines into catechol intermediates that oxidize into the corresponding *o*-quinones. These highly redox active *o*-quinones will subsequently react with GSH, free cysteine and cysteinyl residues on proteins, resulting in significant modification of macromolecules, including proteins, lipids and deoxyribonucleic acid (DNA).

Also, *o*-quinones can enter in oxidative cycling, increasing oxidative stress and depleting antioxidant cell defences and, afterwards, following oxidations of *o*-quinones will lead to the formation of aminochromes, whose subsequent oxidation leads to the production of polymers of melanin type (da Silva et al., 2014a). These pigments have maximal absorbance in the same wavelength range of the formazans formed through MTT reduction and could, therefore, influence the absorbance readings, in the viability assay. Fortunately, this was not problematic since the aminochrome formation only occurred at concentrations higher than those for which the maximum effect in the assay (*i.e.*, 100% cell death) had already been achieved (concentrations higher than 3.68 mM for butylone, 1.47 mM for buphedrone, 10 mM for 6-APB, 5 mM for 5-APB, 0.625 mM for 3,4-DMMC and 10 mM 4-FMC). If this was not the case, to completely rule out possible interference with the viability results, another type of assay would have to be considered for cell death evaluation.

The formation of ROS and RNS is one of the most prominent toxicity mechanisms of amphetamines, both in primary rat hepatocytes and HepG2 cells (da Silva et al., 2014a; Pontes et al., 2008). Along with 4-FMC, 3,4-DMMC (the most toxic substance in the MTT assay) was also responsible for eliciting one of the most significant increases in ROS and RNS at nearly all EC_x tested, in primary hepatocytes. Curiously, 4-FMC was the least toxic compound for primary rat hepatocytes in the viability assay (although in the current determination all drugs were tested at the same effect levels). We expected to observe a higher increase of ROS and RNS for butylone, since this drug has a methylenedioxy group in its structure, which is likely to be degraded into catechol, further forming highly reactive *o*-quinones. Notwithstanding, significant oxidative stress was only evident at concentrations higher than the EC_{50}, suggesting that natural antioxidant defences might be sufficient to protect cells from generated deleterious species. This may also be the case of 5-APB and 6-APB.

One of the most prominent cell defence against reactive species and xenobiotics is GSH. The role of this pivotal antioxidant becomes even more important when considering the liver, which is the drug-metabolizing organ by excellence and, therefore, a first target for damage induced by noxious compounds (Forman et al., 2009; Yuan and Kaplowitz, 2013). In the present work, all the tested stimulants were capable of inducing GSH depletion; this is related to an increased production of reactive species, but this effect was not always accompanied by the complementary expected increase in GSSG levels. Valente and co-workers (2016) also verified this discrepancy between GSH and GSSG levels when evaluating the hepatotoxic potential of few synthetic cathinones. The GSSG efflux into the cell culture medium is promoted to protect cells against excessive oxidative stress, and it has already been hypothesised for amphetamines (Carvalho et al., 1996; da Silva et al.,

2014a). On the other hand, GSH depletion might also be explained by the formation of conjugates with metabolites, resembling what also happens with amphetamines (Carvalho et al., 2012). This hypothesis waits confirmation.

The results obtained herein show that all tested synthetic cathinones and benzofurans were capable of interfering significantly with ATP homeostasis. The dysfunction of normal cell energetics interferes with cell viability since ATP is pivotal for cell survival. The capacity to damage normal ATP production has also been verified for MDMA, which can induce concentration- and time-dependent ATP depletion in rat hepatocytes (Carvalho et al., 2012). Regarding beta-keto-amphetamines hepatotoxicity, Valente and co-workers (2016) also registered a decrease in ATP after exposure of isolated rat hepatocytes to the drugs.

The concentrations of NPS tested here were higher than the concentrations that could be quantified in blood sample of an intoxicated patient (Chan et al., 2013; McIntyre et al., 2015; Thornton et al., 2012; Usui et al., 2014; Zuba et al., 2013). However, it is well-known that liver is exposed to concentrations of amphetamine-like stimulants far higher than the concentrations achieved in blood. Considering the aforementioned structural and functional similarities between these compounds, it is possible that the same occurs with the drugs tested in this work.

Conclusion

Herein it was demonstrated for the first time the concentration-dependent hepatotoxic effects of four synthetic cathinones (butylone, buphedrone, 3,4-DMMC and 4-FMC) and two benzofurans (6- and 5-APB) in three *in vitro* models: the human cell lines HepG2 and HepaRG, and the primary rat hepatocytes. For all models, 3,4-DMMC proved to be the most hepatotoxic compound and primary rat hepatocytes displayed highest sensitivity to all the utilized *in vitro* models. Our findings suggest that the formation of intermediate o-quinones may be involved in the metabolism and hepatotoxicity of the tested NPS. Synthetic cathinones and benzofurans are marketed without previous testing for human safety and wrongly assumed to be safer than classical stimulants. Notwithstanding, we demonstrated that all drugs were capable of inducing oxidative stress and depleting ATP in primary rat hepatocytes; these toxic mechanisms overlap those for amphetamines and other synthetic cathinones. It is important to conduct further studies, which include the investigation of the toxic potential of the metabolites of the tested drugs, to better understand the full extent of the risks to which consumers of these NPS are exposed to.

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